

# Differential expression of endometrial integrins and progesterone receptor during the window of implantation in normo-ovulatory women treated with clomiphene citrate

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**Objective:** To assess the effect of clomiphene citrate (CC) on endometrial epithelial integrins and P receptors (PR) during the window of implantation.

**Design:** Controlled, prospective, clinical study.

**Setting:** Teaching hospital and university research laboratory.

**Patient(s):** Thirty-one fertile, normo-ovulatory women participated in this trial. Thirteen women exhibited a CC-stimulated cycle with 50 mg on days 5–9, and 18 women with spontaneous menstrual cycles served as controls.

**Intervention(s):** Endometrial biopsies in the midluteal phase.

**Main Outcome Measure(s):** Immunohistochemical determination and endometrial cellular localization of  $\alpha_1$ ,  $\alpha_v$ ,  $\beta_3$ , and  $\alpha_4$  epithelial integrins and PR during the window of implantation. The staining intensity was assessed by a semiquantitative index (HSCORE) and compared by nonparametric Mann-Whitney test.

**Result(s):** Higher plasma levels of P and E<sub>2</sub> and delayed histologic dating of the endometrium (38%) were features of CC-treated women. In addition, a low epithelial  $\beta_3$  integrin expression and persistent PR were observed in glandular epithelial cells of “out-of-phase” endometrial biopsies from CC-treated women. In contrast, in “in-phase” biopsies, neither epithelial PR nor  $\beta_3$  integrin were different from spontaneous control cycles. There was no difference in the expression of  $\alpha_1$ ,  $\alpha_v$ , and  $\alpha_4$  between the groups studied.

**Conclusion(s):** The administration of clomiphene produces aberrant endometrial  $\beta_3$  integrin expression in conjunction with a failure in the down-regulation of PR during the window of implantation in a significant number of normo-ovulatory women, notwithstanding the higher plasma P levels. Therefore, CC might affect the expression of endometrial receptivity markers.

**Key Words:** Integrins, progesterone receptors, clomiphene citrate, endometrial receptivity

Integrins belong to the cell–cell adhesion family of molecules, which might be involved in implantation mechanisms and have been suggested as molecular markers of endometrial receptivity (1, 2). A number of investigators have demonstrated that glandular and stromal endometrial integrins are expressed in a predictable and cyclical manner during the luteal phase. The  $\alpha_1$ ,  $\alpha_v$ , and  $\beta_3$  integrins are co-expressed in the epithelial endometrium only during days 20–24 of the menstrual cycle, supporting the concept that these cycle-dependent proteins, expressed within the putative implantation window, are potential biochemical markers of uterine

receptivity (3–5). Moreover, we and others have demonstrated that certain infertility conditions, such as unexplained infertility (6, 7), endometriosis (8), hydrosalpinx (9), and luteal phase deficiency (LPD) (10, 11), are associated with an aberrant pattern of integrin expression. It seems that physiologic epithelial P receptor (PR) down-regulation and  $\alpha_v\beta_3$  integrin expression are critical steps in the implantation process. For example, LPD is characterized by inadequate ovarian P secretion, leading to delayed endometrial maturation and a failure in the down-regulation of PR. Both of these events are closely associated with an aberrant low expression of  $\alpha_v\beta_3$  epithelial integrin (12).

Clomiphene citrate (CC) is a compound widely used in ovulation induction. Clomiphene citrate is a mixture of cis and trans isomers with estrogenic and antiestrogenic effects. It binds to the estrogen receptors (ER), leading to the misinterpretation of the E<sub>2</sub> feedback mechanism at the hypothalamic level. The administration of this drug results in increased pituitary gonad-

Supported by Re-entry Grant 2001 from Programa Latinoamericano de Capacitación e Investigación en Reproducción Humana, Mexico, and Fondo de Investigación Avanzada en Areas Prioritarias (FONDAP) Grant 15010006, Universidad de Chile, Santiago, Chile.

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otropin release and enhanced follicular development and ovarian response (13, 14).

The inconsistency between high ovulation (60%–85%) and low (30%–40%) pregnancy rates characteristic of CC (15) in anovulatory women has been partially explained by its antiestrogenic effect on cervical mucus and the endometrium. These effects are morphologic, morphometric, and biochemical in nature (16–18).

The endometrial antiestrogenic effect of CC has been proposed as a possible explanation for abnormal endometrial maturation. It is, therefore, tempting to hypothesize that long-lasting ER occupancy by CC (19) might alter the endometrial cell function, thus affecting the expression of proteins related to uterine receptivity.

To investigate whether CC provokes changes in the pattern of endometrial receptivity, endometrial samples were obtained during the midsecretory phase from normo-ovulatory women treated with CC to test the expression of integrin subunits and PR.

## MATERIALS AND METHODS

### Subjects

Endometrial samples were obtained from 31 regularly cyclical parous women (mean parity range 2–5) requesting tubal ligation, who were invited to participate in the study. Only women with regular menstrual cycles were recruited; participants with a history of miscarriage were not included. Thirteen women received CC (50 mg orally) on days 5–9 of the menstrual cycle, and 18 women with spontaneous ovulatory cycles served as controls. Surgery and endometrial biopsies were scheduled at the midsecretory phase, within the 6th to 8th postovulatory day. Ovulation was assessed by the surge of LH in urine (Clear-plan ovulation test; Unipath Limited, Bedford, United Kingdom) and by serial ultrasonography with a 7.5-MHz transvaginal probe (Model SA 6000; Medison, Seoul, Korea). The detection of urinary LH surge was considered as day 0, and ovulation (follicular rupture) was confirmed by loss of clear demarcation of its walls and intrafollicular echoes on vaginal ultrasound.

The Ethics Committee of the San Borja-Arriarán Clinical Hospital, Santiago, Chile, approved the investigation protocol, and all women participating in the study provided written informed consent.

### Radioimmunoassay

Progesterone and E<sub>2</sub> concentrations were determined from plasma samples at the time of the endometrial biopsy and measured by specific RIA, as previously reported (20).

### Tissue Preparation

Endometrial tissue was obtained through a sampling device (Pipelle de Cornier, Paris, France). A portion of tissue samples was immediately embedded in a Tissue-Tek Optimal

Cutting Temperature compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at –80°C until used to determine the integrin expression in a cryostat section. A second portion was fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin at 60°C to form paraffin blocks, which were used for PR immunostaining. Sections of 4 μm were stained with hematoxylin-eosin and evaluated for endometrial dating according to Noyes's criteria (21). The dating of endometrial samples was evaluated independently by two pathologists (F.G. and P.P.) blind to the clinical treatment.

### Immunohistochemistry

Immunostaining of endometrial epithelial integrins was conducted as previously reported, with minimal modifications (22). Briefly, the localization of epithelial integrins was assessed in serial cryostat sections (4–5 μm) on silanized slides fixed in acetone at –20°C for 20 minutes. The immunostaining was performed with the streptavidin-biotin-peroxidase method (DAKO, Carpinteria, CA), and 3-amino-9-ethylcarbazole (AEC [DAKO]) was used as a chromogen. The following specific monoclonal antibodies targeted to integrins were used diluted in PBS–bovine serum albumin (BSA): anti-α<sub>1</sub> (clone TS 2/7, 1:2000), anti-α<sub>4</sub> (clone B5G10, 1:1000), and anti-β<sub>3</sub> (clone SSA6, 1:500). The monoclonal antibodies were generously donated by Professor B. Lessey (University of North Carolina, Chapel Hill, NC), and anti-α<sub>v</sub> (clone VNR 147, 1:200) was obtained from GIBCO, BRL Products, Gaithersburg, MD. Nonspecific staining was minimized by incubation with a blocking solution (LSAB Kit system, DAKO).

Primary antibodies were incubated at 4°C overnight. Negative controls were incubated with irrelevant mouse monoclonal antibodies instead of primary antibodies. A secondary antibody (biotinylated goat antimouse Ig, diluted 1:500 in PBS-BSA 1%) was added after three PBS rinses and followed by 30 minutes of incubation.

The sections were then incubated with streptavidin-biotinylated horseradish peroxidase macromolecular complex for 30 minutes at room temperature, followed by the addition of AEC for 10 minutes to complete the reaction. Finally, samples were washed in water, counterstained with hematoxylin, and mounted in a glycerol-based medium.

Immunohistochemistry for PR was performed on 5–6-μm sections of formalin-fixed, paraffin-embedded endometrial biopsies according to the labeled streptavidin-biotin method (DAKO ER/PR system), as previously described (23). 3,3'-diaminobenzidine (DAB) was used as a chromogen. Tissue sections were deparaffinized in xylene and hydrated in a series of graded ethanols. After heat induction in a water bath (95°C–99°C) for 20 minutes in a target retrieval solution (diluted 1:10 with distilled water), samples were incubated with 3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity.

After incubation with blocking serum (PBS-BSA 1%) for 30 minutes at room temperature, mouse monoclonal antibodies to PR (1A6 DAKO ER/PR system) were added and incubated at room temperature for 30 minutes, according to the manufacturer's indications. The negative control was run with mouse monoclonal IgG1 antibody.

A PBS gentle rinse (pH 7.2–7.6) was followed by incubation with a biotinylated goat antirabbit Ig for 30 minutes. After this incubation, sections were washed and incubated with streptavidin conjugated to horseradish peroxidase in PBS complex for 30 minutes. Staining was completed after 10 minutes of incubation with a freshly prepared substrate–chromogen solution (20  $\mu$ L DAB chromogen per 1 mL of PBS). Finally, sections were counterstained with hematoxylin for 2 minutes, washed with distilled water, and coverslipped over the mounting solution for evaluation by light microscope. Two observers blind to information regarding the endometrial samples independently evaluated the positive staining under a microscope (Nikon, Tokyo, Japan).

Results of integrin staining were scored with a semiquantitative index, HSCORE:  $\sum pi (i + 1)$ , where  $i$  = the intensity of staining, with a value of 1, 2, or 3 (weak, positive, or strong, respectively), and  $pi$  = the percentage of stained epithelial cells, varying from 0 to 100%. Progesterone receptor staining was evaluated as present or absent.

### Statistical Evaluation

The number of subjects in this study was calculated assuming an  $\alpha$  of .05,  $\beta$  of 20%, a difference between means, and an SD appropriate to each pair of means to be compared. When dealing with proportions and small numbers, we used Fisher's exact test to test the significance.

The Kolmogorov test was used to test normality in continuous variables (24). Because this test was negative, a nonparametric Mann-Whitney test was performed to com-

pare means between groups. The  $\alpha$  level selected was equivalent to .05.

Fisher's exact test was used to compare endometrial histologic data (clomiphene/controls  $\times$  in-phase/out-of-phase).

### RESULTS

Table 1 illustrates the clinical characteristics of the participants, including mean age, body mass index (BMI), range of endometrial dates at the time of study, and plasma P and E<sub>2</sub> concentrations in control and CC-treated subjects. There were no significant differences in mean age, parity, and BMI among the subjects studied. As expected, the number of CC–ovulatory follicles (>17 mm) (data not shown) and the levels of P and E<sub>2</sub> plasma were greater at the time of biopsy in the CC-treated group (11.13  $\pm$  1.3 ng/mL and 165.88  $\pm$  12.1 pg/mL, respectively), compared with the spontaneous cycle group (7.13  $\pm$  1.3 ng/mL and 93.33  $\pm$  10.2 pg/mL, respectively) ( $P$  = .0001 and  $P$  = .03). High endometrial delayed maturation ( $\geq$ 3 days) was observed in 38.4% of the CC-treated group compared with 16.6% in the spontaneous cycle group ( $P$  = .031). Glandular–stromal desynchronization and high stromal edema were the most frequent histologic features observed in “out-of-phase” CC endometria (Fig. 1D).

Table 2 summarizes the endocrine endometrial histology and immunohistochemical data of spontaneous and CC-stimulated cycles during the midluteal phase. The plasma P levels observed in “in-phase” endometria were similar in control and CC-stimulated subjects. However, neither P nor E<sub>2</sub> plasma concentrations were different between “out-of-phase” (13.52  $\pm$  2 ng/mL and 170.8  $\pm$  15.2 pg/mL, respectively) and “in-phase” endometria (9.23  $\pm$  1.24 ng/mL and 171  $\pm$  17.4 pg/mL, respectively) of CC-stimulated cycles. In contrast, luteal plasma P concentrations were significantly lower in control participants ( $n$  = 3) with an “out-of-phase” endometrium compared with CC-stimulated cycles ( $P$  = .01).

**TABLE 1**

**Clinical and endocrine characteristics of CC-treated subjects and spontaneous cycle controls during the implantation window.**

Characteristics	Spontaneous cycle (n = 18)	CC-treated (n = 13)	P
Age (y)	34.6 $\pm$ 1.3	37.6 $\pm$ 0.9	NS
Parity	3.1 $\pm$ 0.2	3.0 $\pm$ 0.2	NS
BMI	24.8 $\pm$ 0.9	27.1 $\pm$ 0.7	NS
E <sub>2</sub> (pg/mL)	93.3 $\pm$ 10.2	165.8 $\pm$ 12.1	<.05 <sup>a</sup>
P (ng/mL)	7.1 $\pm$ 1.3	11.1 $\pm$ 1.3	<.05 <sup>a</sup>
Delayed endometrium, n (%)	3 (16.6)	5 (38.4)	

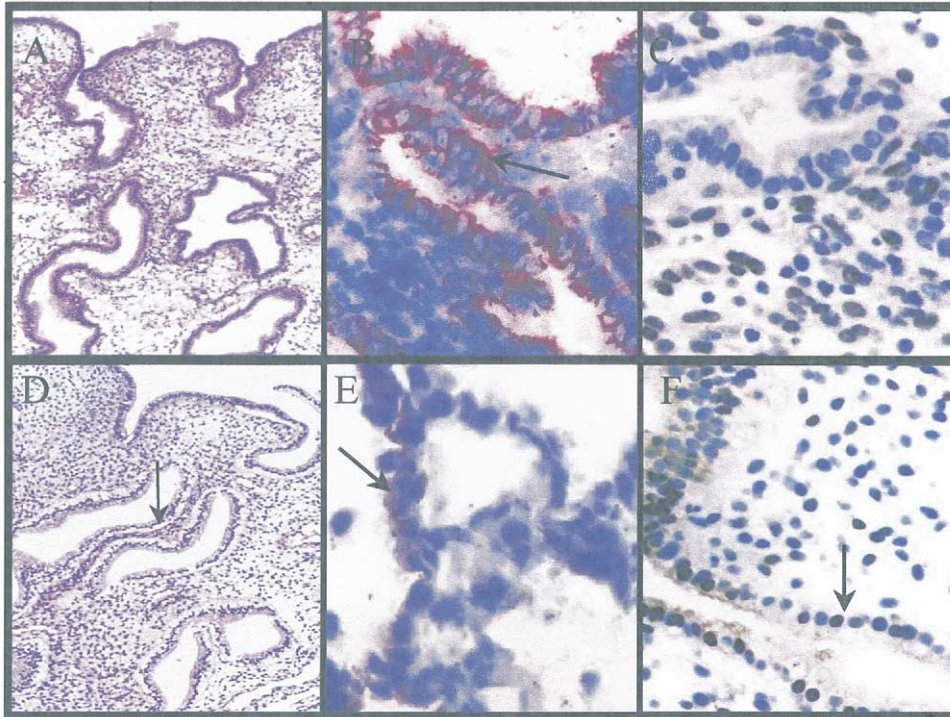
Note: Values are means  $\pm$  SEM, unless otherwise noted. CC = clomiphene citrate; BMI = body mass index; NS = not significant.

<sup>a</sup>Clomiphene-treated endometrium vs. spontaneous endometrium.



## FIGURE 1

Hematoxylin-eosin (H & E) staining and immunohistochemical localization of  $\beta_3$  integrin and PR in endometrial biopsies obtained during the window of implantation from normal (A, B, C) and clomiphene-treated subjects (D, E, F). (A) Normal “in-phase” endometrium (H & E staining). (B) Arrow depicts intense immunostaining of  $\beta_3$ . (C) Absent PR in glands of normal spontaneous biopsy. (D) Desynchronized endometrial development in “out-of-phase” CC-endometrium (H & E staining). Arrow depicts subnuclear vacuoles corresponding to early postovulatory endometrium. (E) Arrow indicates moderate intensity of  $\beta_3$  staining. (F) Arrow indicates persistent PR in glandular epithelium of a CC-treated subject. Original magnification,  $\times 10$  in A,  $\times 20$  in D,  $\times 40$  in B, C, E, F.



Palomino. Clomiphene and endometrial receptivity. *Fertil Steril* 2005.

Interestingly, staining intensity for  $\beta_3$  integrin was lower in both types of “out-of-phase” endometria, indicating mechanisms governing endometrial histology distinct from  $\beta_3$  integrin expression in the presence of CC.

In addition, persistent epithelial staining for PR was a feature of an “out-of-phase” endometrium in 38% of CC-treated women, despite higher levels of plasma P. These data suggest that in certain women, endometrial PR is differentially regulated by CC administration.

Figure 1 shows the light microscopic features and the immunohistochemical identification of the  $\beta_3$  integrin subunit and PR within endometrial tissue collected during the window of implantation in spontaneous and CC-stimulated cycles. A strong staining of the  $\beta_3$  integrin subunit was observed in glands of “in-phase” endometrial tissue collected from spontaneous cycle women during the midsecretory phase (Fig. 1B). In contrast, the  $\beta_3$  integrin subunit was under-expressed (Fig. 1E) in epithelial endometrial cells

from CC-treated women with “out-of-phase” endometrial tissue.

Nuclear PR was strongly stained in the stromal cells and was completely absent in epithelial and glandular cells (Fig. 1C). This pattern of expression was observed in normal midluteal phase endometria and “in-phase” CC-treated biopsies. Unlike a normal endometrium, the staining intensity of nuclear PR persists in the glandular epithelium of a delayed endometrium in CC-treated cycles (Fig. 1F) and spontaneous “out-of-phase” endometrium (data not shown).

Collectively, a greater immunostaining for  $\beta_3$  integrin and down-regulation of PR are characteristic of an “in-phase” endometrium from spontaneous and CC-stimulated cycles during the window of implantation. In contrast, a significant reduction in the expression of  $\beta_3$  integrin and the presence of PR are features of “out-of-phase” endometria during the midluteal phase.

TABLE 2

Endometrial dating, plasma E<sub>2</sub> and P concentrations, and β<sub>3</sub> integrin and PR endometrial expression from clomiphene and spontaneous cycles during the implantation window.

	Spontaneous endometrium (n = 18)		CC-treated endometrium (n = 13)		P
	"In-phase"	"Out-of-phase"	"In-phase"	"Out-of-phase"	
Subjects, n (%)	15 (83)	3 (17)	8 (61.5)	5 (38.4)	
E <sub>2</sub> (pg/mL)	93.5 ± 11	71.3 ± 2.5	171 ± 17.4	170.8 ± 15.2	NS
P (ng/mL)	7.86 ± 1.52	4.57 ± 0.9 <sup>a</sup>	9.2 ± 1.24	13.5 ± 2 <sup>a</sup>	<.05 <sup>a</sup>
β <sub>3</sub> integrin (HSCORE)	2.8 ± 0.09	1.6 ± 0.3 <sup>b</sup>	2.59 ± 0.2	1.45 ± 0.2 <sup>b</sup>	<.05 <sup>b</sup>
PR	Absent	Present	Absent	Present	

Note: Values are means ± SEM, unless otherwise noted. CC = clomiphene citrate; PR = P receptor.

<sup>a</sup>"Out-of-phase" vs. "in-phase" from spontaneous endometrium.

<sup>b</sup>"In-phase" vs. "out-of-phase" from both spontaneous and clomiphene-treated endometrium.

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Figure 2 illustrates the semiquantitative evaluation of the integrin subunits (HSCORE) within endometrial tissue during the midsecretory phase of spontaneous and CC-stimulated cycles.

The β<sub>3</sub> integrin expression was significantly greater in normal ovulatory subjects compared with CC-treated subjects ( $P=.012$ ). The staining intensity of α<sub>1</sub>, α<sub>v</sub>, and α<sub>4</sub> integrins was comparable at the midluteal phase in control and CC-treated subjects. However, the α<sub>1</sub> integrin exhibited a greater staining intensity, whereas α<sub>v</sub> and α<sub>4</sub> had a low expression in both groups studied. Interestingly, no statistical differences were observed when comparing α<sub>1</sub>, α<sub>v</sub>, and α<sub>4</sub> HSCORE from "in-phase" and "out-of-phase" endometria.

## DISCUSSION

Implantation involves a complex interaction between the implanting embryo and the maternal endometrium. To prepare for implantation, the endometrium undergoes a precise developmental program during the follicular phase under the control of follicular E<sub>2</sub>. After ovulation, the endometrium exhibits specialized secretory changes governed by luteal P secretion (25). In mammals, a number of specific molecules have been implicated as critical factors for embryonic implantation. These factors include leukemia inhibitory factor, epidermal growth factor, integrins, and PR. In humans, the integrins are one of the most characterized markers for uterine receptivity. However, the underlying mechanisms controlling their expression within endometrial tissue and their exact role in the implantation process are not completely understood (26, 27).

It is also thought that epithelial PR down-regulation is a critical molecular event in endometrial receptivity. It seems that PR down-regulation allows the expression of epithelial endometrial proteins such as α<sub>v</sub>β<sub>3</sub> integrin, which is regulated by transforming growth factor-α and epidermal growth factor pro-

duced by endometrial stromal cells in a paracrine fashion. The failure in the down-regulation of PR is closely associated with aberrant α<sub>v</sub>β<sub>3</sub> integrin expression leading to an infertile condition, such as LPD (28). In addition, the reappearance of ER and PR was detected within the endometrial epithelium after PR antagonist administration, suggesting that PR down-regulation is a P-dependent process (29).

The discrepancy between ovulation and the low pregnancy rates in infertile patients treated with CC is well known, and several hypotheses have been proposed: endocrine effect diminishes cervical mucus-sperm interaction and delayed endometrial development (30). It has been hypothesized that the competitive, long-time ER occupancy by CC might disrupt endometrial cell function. Thus, the present investigation extends our knowledge concerning the expression of critical endometrial proteins at the time of implantation, including integrin subunits and epithelial PR in fertile normo-ovulatory women treated with CC.

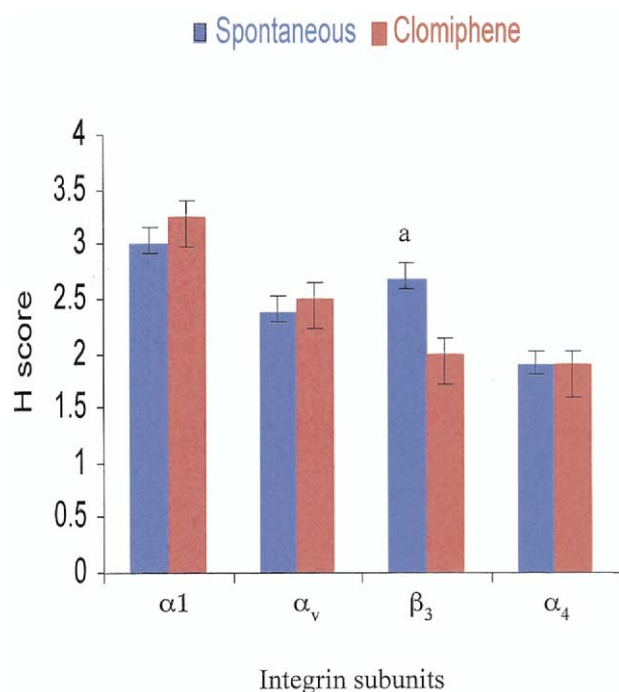
To avoid blind causes of infertility that might interfere with the development and expression of a receptive endometrium, only ovulatory fertile women were invited to participate in this clinical study. Our data, collected at the time of the window of implantation, indicate that there were no differences in the plasma concentrations of E<sub>2</sub> and P between "out-of-phase" and "in-phase" endometria. These findings corroborate a previous study suggesting that plasma concentrations of steroids are independent from endometrial development in CC-treated cycles (31).

The failure in the down-regulation of PR and aberrant β<sub>3</sub> epithelial integrin expression were observed in nearly 40% of the endometria examined in CC-treated women. This might reflect altered cell function, presumably caused by the local antiestrogenic effect of CC (32, 33).

The present study examines the expression of the α<sub>v</sub> integrin subunit in midluteal phase endometria from sponta-

## FIGURE 2

Immunohistochemical staining evaluation by HSCORE of epithelial endometrial integrin subunits from normal and clomiphene-treated biopsies obtained during the window of implantation. Results are means  $\pm$  SEM. <sup>a</sup> $P=.012$  between normal spontaneous and clomiphene-treated women.



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neous and CC-stimulated cycles, and our current findings indicate no difference in the expression of the  $\alpha_v$  integrin subunit, results similar to those from previous observations in unexplained infertile patients stimulated with CC (34).

It is well known that  $\alpha_1$  integrin subunit expression arises at the time of ovulation and persists throughout the luteal phase. Interestingly, our findings indicate that  $\alpha_1$  integrin subunit expression was greatest in the endometrial epithelium of both spontaneous and CC-stimulated cycles. The low expression of the  $\alpha_4$  integrin subunit in CC-treated and spontaneous samples determined in our study confirms previous reports advocating the notion that this subunit appears sporadically and that its absence does not represent an endometrial dysfunction (35).

It is possible that the long-time ER occupancy by CC might alter the cellular response and function of endometrial cells, which is consonant with inadequate steroid receptor induction. This might explain our findings of abnormal PR modulation during the midsecretory phase, notwithstanding the high P plasma concentration. However, other studies did

not find differences in steroid receptor concentration in the endometria of CC-treated women. One possible explanation for these discrepancies might be found in the use of different methodologies to assess endometrial PR, such as steroid-binding assay (36) or enzyme-linked assay (37).

Our immunohistochemical studies determined the cellular localization of PR protein within the midluteal endometrium without biochemical studies that need to homogenize the tissue. Collectively, our results suggest that the mechanism responsible for the changes in endometrial receptivity in CC-treated cycles is different from LPD. Failure in the down-regulation of endometrial PR in the midluteal phase is a feature of LPD associated with diminished P production, resulting in "out-of-phase" endometrial maturation.

Interestingly, in this study we show that P plasma concentrations are higher in CC-treated women, and there are no differences between  $E_2$  and P plasma concentrations in delayed and "in-phase" endometria in the CC-stimulated subjects. These data support our hypothesis that the local effect of CC plays a key role in these morphologic and molecular changes. In conclusion, we observed that CC administration to normo-ovulatory women provokes delayed secretory endometrial maturation and persistent epithelial PR, closely associated with a reduced  $\beta_3$  epithelial endometrial integrin expression in a high proportion of endometrial tissue (38%), which is significantly greater compared with the delayed endometrial dating revealed in control subjects.

The reduction of the immunohistochemical staining for  $\beta_3$  integrin and the persistence of PR in epithelial endometrial cells might account for low accumulated pregnancy rates in CC-ovulatory subjects. These observations support the notion of controversial clinical results of CC administration to ovulatory women.

*Acknowledgments:* The authors thank Professor Bruce A. Lessey (University of North Carolina, Chapel Hill, North Carolina) for providing the monoclonal antibodies anti- $\alpha_1$ , - $\alpha_4$ , and - $\beta_3$  integrin subunits, and Paulina Peñaloza, M.D., from the Department of Pathology, University of Chile, Santiago, for histologic dating evaluation.

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